

# Specimen Sample Preservation for Cell and Tissue Cultures

Gabrielle Meeker, Karolyn Ronzano, Karen Scribner, and Robert Evans

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Space Administration



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## INTRODUCTION

The era of the International Space Station with its longer duration missions will pose unique challenges to microgravity life sciences research. The Space Station Biological Research Project (SSBRP) is responsible for addressing these challenges and defining the science requirements necessary to conduct life science research on-board the International Space Station. Space Station will support a wide range of cell and tissue culture experiments for durations of 1 to 30 days. Space Shuttle flights to bring experimental samples back to Earth for analyses will only occur every 90 days. Therefore, samples may have to be retained for periods of up to 60 days. This presents a new challenge in fresh specimen sample storage for cell biology. Fresh specimen samples are defined as samples that are preserved by means other than fixation and cryopreservation. The challenge of long-term storage of fresh specimen samples includes the need to suspend or inhibit proliferation and metabolism pending return to Earth-based laboratories. With this challenge being unique to space research, there have not been any ground based studies performed to address this issue. It was decided by SSBRP that experiment support studies to address the following issues were needed, Fixative Solution Management; Media Storage Conditions; Fresh Specimen Sample Storage of Mammalian Cell/Tissue Cultures; Fresh Specimen Sample Storage of Plant Cell/Tissue Cultures; Fresh Specimen Sample Storage of Aquatic Cell/Tissue Cultures; and Fresh Specimen Sample Storage of Microbial Cell/Tissue Cultures. The objective of these studies was to derive a set of conditions and recommendations that can be used in a long duration microgravity environment such as Space Station that will permit extended storage of cell and tissue culture specimens in a state consistent with zero or minimal growth, while at the same time maintaining their stability and viability. The first two issues, Fixative Solution Management and Media Storage Conditions, were studied by the SSBRP science staff at Ames Research Center. The final results of these studies are presented in this report. The remaining four issues examining fresh specimen sample storage were subcontracted to external organizations for investigation. Included in this report is a list of these studies, the investigators, the scope of their study, and a contact for the results.



## **EXTRAMURAL SCIENCE REQUIREMENTS DEFINITION STUDIES**

## **FRESH SPECIMEN SAMPLE STORAGE OF MAMMALIAN CELL/TISSUE CULTURES**

### **Performing Organization**

American Type Culture Collection

### **Principal Investigator**

Dr. Robert Hay

### **Study Scope**

ATCC defined conditions necessary for the storage of 14 different human and animal cell lines at temperatures of 4°C, 10°C, and 20°C for up to 40 days. Methods in use currently for shipping living cells in culture flasks promote excellent survival of most cell lines for periods of 7-10 days at ambient temperatures (20-25°C). The extension of this period with concomitant retention of cell viability and function was attempted by ATCC with the understanding that the goal was to arrest cell growth and cycling. Standard measurements for survival were utilized, namely cell viability, which was determined by dye exclusion, clone forming efficiencies, morphologic observation, Sister Chromatid Exchange, and biochemical analyses. The ability of cells to exhibit characteristic function immediately after recovery from hypothermic storage, with and without stabilizing agents, was assessed.

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### **Performing Organization**

Kansas State University

### **Principal Investigator**

Dr. Terry Johnson

### **Study Scope**

Dr. Terry Johnson's laboratory at KSU investigated the potential ability of a unique cell cycle inhibitor to hold cells in an arrested and viable state for extended periods. The purified inhibitor, Cell Regulatory Sialoglycoprotein (CeReS), already had been shown to induce a reversible cell cycle arrest in which cellular viability has been maintained for at least 2 weeks. Stabilization studies were performed on three mammalian cell lines and two tissue culture explants. Serum downshifts, temperature downshifts, and the application of CeReS were tested.

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## **FRESH SPECIMEN SAMPLE STORAGE OF PLANT CELL/TISSUE CULTURES**

### **Performing Organization**

State University of New York

### **Principal Investigator**

Dr. Abraham Krikorian

### **Study Scope**

This research effort identified conditions for the maintenance and stabilization of well-characterized cultures. Cultures were selected that would provide excellent model systems for a) somatic embryogenic and organogenic development (carrot, orange, and daylily) and for b) biosynthetic potential (vinca and soybean). Comparative studies were carried out to ascertain efficient strategies for their "zero growth" or "zero developmental progression" maintenance. Preliminary tests using the proposed methods were conducted to define areas of greatest promise and then focus was placed on those of greatest merit. As non-invasive a method or combination of methods as possible was used. Cell cultures were examined for cellular integrity, growth, and retention of metabolic capability.

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### **Performing Organization**

Washington State University

### **Principal Investigator**

Dr. Norman Lewis

### **Study Scope**

This study proposed to identify conditions for the maintenance and stabilization of well-characterized cultures. These cultures were selected to provide excellent model systems to examine questions of stabilization/viability. Comparative experiments were undertaken with species proposed for space-flight studies. Cell cultures were examined for cellular integrity, metabolism, protein and cDNA expression, marker enzymes and by electron microscopy. The goal was to return cell cultures to normal maintenance medium and examine them for viability/integrity.

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## **FRESH SPECIMEN SAMPLE STORAGE OF PLANT CELL/TISSUE CULTURES**

### **Performing Organization**

University of Colorado (BioServe)

### **Principal Investigator**

Dr. Paul Todd

### **Study Scope**

This study proposed to investigate biophysical and biological methods for maintaining plant cultures in a state of quiescence for a period of at least 40 days. The viability of the cells were evaluated at appropriate assessment intervals of not less than 7 days. Studies included stabilization of plant cell and tissue cultures under various temperatures, and osmotic inhibitions. The methods were performed on well-characterized plant cultures that are suited for spaceflight. Specific preservation techniques that indicated a high degree of success, were tested additionally.

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## **FRESH SPECIMEN SAMPLE STORAGE OF MICROBIAL CELL/TISSUE CULTURES**

### **Performing Organization**

American Type Culture Collection

### **Principal Investigator**

Dr. Francis Molina

### **Study Scope**

ATCC investigated the long term storage of 5 to 10 microbial strains at various temperatures using additives likely to be successful stabilizers. Cultures initially grown at normal growth temperatures were gradually cooled to 3 different holding temperatures in the presence of various stabilizing additives. Cell viability was assayed periodically for up to but not limited to 40 days using an Alamar Blue assay which is an indicator of cellular metabolic activity. The following vital fluorescent probes (Molecular Probes, Inc., Eugene, OR) were evaluated: 1) Live/Dead *FungoLight*, 2) Live/Dead *BacLight*, and 3) Syto live cell nucleic acid stains. Results of viability assays were correlated with most probable numbers (MPN) or colony counts. Generation times and multi-locus DNA fingerprints of cells derived from those treatments yielding the best viability assays were examined to assess the effects of exposure to the protectants

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### **Performing Organization**

Kansas State University

### **Principal Investigator**

Dr. Terry Johnson

### **Study Scope**

KSU investigated the long term storage of two species of bacteria, a unicellular yeast, and two species of ciliate protozoa, at various temperatures. The potential stabilization of bacterial cultures, for an extended duration, was investigated by several methods including carbon source deprivation, chloramphenicol arrest, and the use of metabolic inhibitors. The potential stabilization of yeast cultures, for an extended duration, were investigated by a unique and naturally occurring mediator of cell cycle arrest that already has been shown to reversibly stabilize yeast cell cultures for at least short durations, as well as being able to function in the microgravity environment. The feasibility of maintaining viable protozoan cultures for an extended period of time was investigated by temperature reduction and/or the addition of one of several arrestive adrenergic antagonists. KSU investigated the relative feasibility of these methods in maintaining viability of microbial cultures up to but not limited to 40 days and at holding temperatures in the range of 4°C-25°C.

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## **FRESH SPECIMEN SAMPLE STORAGE OF AQUATIC CELL/TISSUE CULTURES AND NON-FEEDING AQUATIC SPECIMENS**

### **Performing Organization**

American Type Culture Collection

### **Principal Investigator**

Dr. Thomas Nerad

### **Study Scope**

This study investigated the long term storage of 5 to 10 unicellular eukaryotic strains at various temperatures. Cultures initially grown at normal growth temperatures were gradually cooled to 3 different holding temperatures in the presence of various stabilizing additives. Cell viability was assayed periodically for up to but not limited to 40 days using an Alamar Blue assay which is an indicator of cellular metabolic activity. Results of viability assays were correlated with direct microscopic/ colony counts. Generation times and multi locus DNA fingerprints of cells derived from those treatments yielding the best viability were examined to assess any effects of exposure to the protectants.

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### **Performing Organization**

University of Colorado (BioServe)

### **Principal Investigator**

Dr. Louis Stodieck

### **Study Scope**

This study investigated biophysical and biological methods for maintaining suspension, tissue, and embryonic cultures in a state of quiescence for at least 40 days. The viability of the cell was evaluated at 10 day assessment intervals. Studies included stabilization of aquatic cell and tissue cultures and aquatic embryos using temperature and viscosity variations, gas partial pressure control, nutrient reduction, and growth inhibitors. The methods were performed on well-characterized aquatic cells, tissues, and multi-cellular organisms that are suited for spaceflight. Temperature profiles were carefully controlled using previously flown flight qualified hardware.

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## **INTRAMURAL SCIENCE REQUIREMENTS DEFINITION STUDIES**



**MEDIA STORAGE CONDITIONS**  
**FINAL REPORT**



## **Media Storage Conditions Final Report**

### **1.0 PURPOSE**

The purpose of this final report is to document the experimental activities conducted and the results obtained and to formulate a recommendation concerning temperature storage conditions required to support long-term media stability. The objective of this study was to determine the shelf life of commonly used basal media and nutritive supplements when stored at 4°C, 12°C, 22°C, or 37°C. The storage conditions, duration, and temperatures that maintain the shelf-life of media were determined by bioassay analysis, and by measuring the pH and glucose content of the media. Bioassay analysis consisted of assessing the cell growth-promoting ability of media as indicated by three endpoints: culture confluence, cell morphology, and mitochondrial metabolism of cultures grown in the various test media. The need for this information arises from the limited electrical power and cold storage space available within the Cell Culture Unit or Space Station for long-term (up to 12 weeks) storage of media and supplements. This study was performed by Martin Marietta Services at Ames Research Center.

### **2.0 GOALS**

- 1) To assess the cell growth-promoting ability of basal media and supplements when stored mixed in solution at 4°C, 12°C, 22°C, or 37°C for up to 12 weeks.
- 2) To assess the cell growth-promoting ability of basal media and supplements when stored separately at 4°C, 12°C, 22°C, or 37°C for up to 12 weeks.
- 3) To assess whether the storage temperature of glutamine, fetal bovine serum, and/or basal media is the critical element in determining the cell growth-promoting ability of complete media. Complete media is defined as basal media plus one or more nutritive supplements.

### **3.0 VARIABLES**

#### **3.1 Basal Media and Supplements**

Basal Media:

Dulbecco's Modified Eagles Medium (DMEM)

Glutamax-Dulbecco's Modified Eagles Medium (G-DMEM; contains a more stable form of glutamine)

Supplements:

Glutamine (1:100 in basal media)

Fetal Bovine Serum (FBS; 10% in basal media)

Basal media contains salts, glucose, and amino acids necessary for basic cell culture maintenance. Most mammalian cell cultures require various supplements, such as serum and/or additional amino acids, to the basal media in order for cell cultures to proliferate and differentiate properly *in vitro*. Fetal bovine serum and glutamine are two common media supplements used to culture human fibroblast cells in the laboratory.

### 3.2 **Experimental Groups**

The following 16 experimental groups were used to assess the cell growth-promoting ability of basal media and supplements, stored separately or mixed in solution, at various test temperatures as described below (Goals #1 and #2).

- 1) DMEM, FBS, glutamine (stored mixed in solution at 4°C)
- 2) DMEM, FBS, glutamine (stored mixed in solution at 12°C)
- 3) DMEM, FBS, glutamine (stored mixed in solution at 22°C)
- 4) DMEM, FBS, glutamine (stored mixed in solution at 37°C)
  
- 5) G-DMEM, FBS (stored mixed in solution at 4°C)
- 6) G-DMEM, FBS (stored mixed in solution at 12°C)
- 7) G-DMEM, FBS (stored mixed in solution at 22°C)
- 8) G-DMEM, FBS (stored mixed in solution at 37°C)
  
- 9) DMEM, FBS, glutamine (stored separately at 4°C and mixed immediately prior to inoculation with fibroblast cells)
- 10) DMEM, FBS, glutamine (stored separately at 12°C and mixed immediately prior to inoculation with fibroblast cells)
- 11) DMEM, FBS, glutamine (stored separately at 22°C and mixed immediately prior to inoculation with fibroblast cells)
- 12) DMEM, FBS, glutamine (stored separately at 37°C and mixed immediately prior to inoculation with fibroblast cells)
  
- 13) G-DMEM, FBS (stored separately at 4°C and mixed immediately prior to inoculation with fibroblast cells)
- 14) G-DMEM, FBS (stored separately at 12°C and mixed immediately prior to inoculation with fibroblast cells)
- 15) G-DMEM, FBS (stored separately at 22°C and mixed immediately prior to inoculation with fibroblast cells)
- 16) G-DMEM, FBS (stored separately at 37°C and mixed immediately prior to inoculation with fibroblast cells)

The following 6 experimental groups were used to assess whether the storage temperature of glutamine, fetal bovine serum, and/or basal media is the critical element in determining the shelf life of complete media (Goal #3). Media and supplements were mixed immediately prior to inoculation with fibroblast cells.

- 17) DMEM (stored at 4°C), FBS (stored at 4°C), glutamine (stored at 12°C)
- 18) DMEM (stored at 4°C), FBS (stored at 4°C), glutamine (stored at 22°C)
- 19) DMEM (stored at 4°C), FBS (stored at 4°C), glutamine (stored at 37°C)
  
- 20) DMEM (stored at 4°C), FBS (stored at 12°C), glutamine (stored at 4°C)
- 21) DMEM (stored at 4°C), FBS (stored at 22°C), glutamine (stored at 4°C)
- 22) DMEM (stored at 4°C), FBS (stored at 37°C), glutamine (stored at 4°C)
  
- 23) DMEM (stored at 12°C), FBS (stored at 4°C), glutamine (stored at 4°C)
- 24) DMEM (stored at 22°C), FBS (stored at 4°C), glutamine (stored at 4°C)
- 25) DMEM (stored at 37°C), FBS (stored at 4°C), glutamine (stored at 4°C)

### 3.3 Sampling Frequency

Each experimental group was stored in duplicate containers with duplicate samples drawn from each container 8 times throughout the study period: prior to storage, at weeks 2, 3, and 4, and at 2 week intervals thereafter for up to 12 weeks. An additional sampling day at week 3 was added to the test plan to better define the changes which occur during the first 30 days of the study, which is the required experimental period of the Cell Culture Unit on-orbit.

## 4.0 **BIOASSAY ANALYSIS**

The ability of media and supplements to support cell growth after storage at various temperatures was assessed using human fibroblast cells, a commonly used cell type in cell and molecular biology. Fibroblast cells are found in connective tissue throughout the body. They are often used as a model system to study gene regulation and were recently shown to respond to a mechanical environment by contracting (B. Johnson-Wint, R. Grymes, and A. Malouvier. 1994. Collagen contraction by corneal, skin and tendon fibroblasts: Mechanisms and force effects. Abstract at the American Society for Cell Biology Thirty-fourth Annual Meeting, San Francisco, CA.)

The specific criteria used were the ability of the media to promote normal cell proliferation, as determined by estimation of culture confluence, and observation of cell morphology. Originally, only one method of assessment was to be used, the MTT (Dimethylthiazol diphenyltetrazolium bromide; Thiazolyl blue; Sigma, St. Louis, MO) assay, which measures the mitochondrial activity of living cells as an indirect measure of cell density. The MTT kit is a colorimetric assay based on the ability of active mitochondrial dehydrogenases of living cells to cause the cleavage of a soluble yellow dye converting it to insoluble purple formazan which absorbs at 570 nm. However, this assay was found to be highly variable in some cases, so the decision was made to supplement this method with two additional analytical techniques: (1) visual determination of culture confluence or the % of the vessel bottom which is covered by cells and (2) visual assessment of cell morphology by photomicroscopy.

Each of the 25 experimental groups of media described above was inoculated with fibroblast cells into 4 wells (1 ml each) of a 24-well cell culture plate. The pH and glucose content of each experimental group were determined prior to plating cells. Cell cultures were allowed to grow for 4 days at 37°C in a 5% CO<sub>2</sub> incubator. After 4 days in culture the confluence, morphology, and absorbance in the MTT assay were determined. Under standard laboratory conditions in which media is stored at 4°C, and FBS and glutamine are stored frozen, culture confluence is 100% after 4 days of growth under the bioassay conditions described above.

Ability of the media types to support fibroblast cell growth after storage at various temperatures was analyzed according to pass/fail criteria. **Failure was defined as less than 75% culture confluence and/or evidence of abnormal cell morphology.**



## 5.0 RESULTS

Results are shown through week 12 for culture confluence, cell morphology, absorbance in the MTT assay, glucose content and pH of complete media. Computer scans of select photomicrographs are included to demonstrate normal compared to abnormal cell morphology. (Figures 1-6)

### 5.1 Confluence and Morphology

#### 5.1.1 Components stored mixed in solution

The effect of storing basal media and supplements mixed in solution on subsequent confluence is shown in Figure 1A. Storage at 4°C, 12°C, or 22°C was associated with 75% confluence or greater through week 6, which encompasses the first 30 days. This is the most critical period in terms of experiment support within the Cell Culture Unit. However, by week 8, only media stored at 4°C had acceptable confluence levels. From weeks 10 to 12, none of the experimental groups had confluence levels greater than 50%. The photomicrographs taken of cultures as early as week 3 (Figure 2; please compare scans within left and right columns) demonstrate that storage at 37°C results in abnormal cell morphology compared to storage at 4°C and 22°C.

#### 5.1.2 Components stored separately

In general, separate storage of media and supplements was not as effective as mixed storage at supporting culture growth. Figure 1B shows that storage at 4°C, 12°C, and 22°C resulted in acceptable or near acceptable confluence levels on weeks 2 and 4. After week 4, confluence fell to unacceptable levels even when media and components were stored at 4°C, and from this point through week 12 none of the groups had confluence levels above 60%. Cell morphology showed the same pattern as above with media stored at 37°C producing abnormal cell morphology compared to storage at 4°C-22°C (not shown).

#### 5.1.3 Components stored separately, differential temperature

Figure 1C shows that separate storage of media and supplements with one component stored at a different temperature than the other two was less effective at preserving shelf-life than storage mixed in solution (Figure 1A) or separate storage at the same temperature (Figure 1B). Only at weeks 3 and 4 did some of the experimental groups show culture confluence at acceptable or near acceptable levels. All other storage times produced confluence levels below 75% for all of the media groups tested. The photomicrographs taken of cultures during week 3 (Figure 3, please compare left and right scan of each pair) demonstrate that glutamine and DMEM are more temperature sensitive than FBS; storage of glutamine and DMEM at 37°C results in abnormal cell morphology compared to storage at 12°C.

## 5.2 Absorbance in the MTT assay

### 5.2.1 Components stored mixed in solution

Absorbance in the MTT assay is an indication of mitochondrial metabolism. A trend toward increasing absorbance in the MTT assay was observed as storage duration increased up to week 8 after which levels appeared to fall off slightly (Figure 4A). This upward trend may reflect greater activity of existing mitochondria and/or increased numbers of new mitochondria.

### 5.2.2 Components stored separately

The pattern observed was similar to that described above, with absorbance increasing as storage duration increased, except for the latter four groups which contained Glutamax-DMEM rather than DMEM supplemented with glutamine (Figure 4B). These groups appeared to peak at week 8 after which absorbance decreased. Maximum absorbance was higher when media components were stored separately compared to when components were stored mixed in solution (Figure 4B versus Figure 4A).

### 5.2.3 Components stored separately, differential temperature

A similar pattern of increasing absorbance with increasing storage duration was observed (Figure 4C). As in Figures 4A and 4B absorbance tended to peak before the end of the study, in this case absorbance peaked at week 10 and then decreased at week 12. Maximum absorbance was higher in these groups than when components were stored mixed in solution (Figure 4A) or stored separately at the same temperature (Figure 4B).

## 5.3 Glucose Content and pH of Stored Media

### 5.3.1 Components stored mixed in solution

Glucose content of the media was variable but did not fall uniformly below initial levels until week 8 after which they appeared to plateau (Figure 5A). Similarly, the pH of the media was stable at initial levels until week 8 after which the pH increased until all of the groups had a pH of 9 at week 12 (Figure 6A).

### 5.3.2 Components stored separately

Glucose content in these groups was measured after mixing the media and supplements and just prior to inoculating the media with cells. The overall trend is toward decreasing glucose content as above and is most pronounced at week 8 after which glucose levels appeared to plateau (Figure 5B). As above, the pH of the media was stable until week 8 after which most of the groups had pH levels of 9 (Figure 6B). The only group which showed maintenance of initial pH levels throughout the 12 weeks of storage was DMEM, FBS, and glutamine stored separately at 4°C.

### 5.3.3 Components stored separately, differential temperature

Glucose content is as variable as in Figures 5A and 5B and showed the same trend towards decreasing glucose levels as storage duration increased, reaching a plateau at week 10 and decreasing slightly at week 12 (Figure 5C). As above, the pH of the media was stable until week 8 after which all but one of the groups had a pH level of 9 (Figure 6C). The exception was DMEM and glutamine stored at 4°C, FBS stored at 12°C which showed maintenance of initial pH levels throughout the 12 weeks of storage.

## 6.0 SUMMARY AND CONCLUSIONS

This study was performed to determine the temperature storage requirements of a common media and supplement mix (Dulbecco's Modified Eagles Medium, Fetal Bovine Serum, and glutamine) used to culture a common mammalian cell type (human fibroblasts). The results obtained here should be relevant to a wide variety of media and cell types likely to be used for research purposes on Space Station.

The results through week 6 of this 12 week study suggest that media and supplements should be stored mixed in solution, rather than in separate containers, at temperatures between 4°C and 22°C in order to promote normal cell proliferation and morphology when subsequently used to culture cells. Morphological observations did suggest that glutamine and DMEM are more temperature sensitive than FBS, however, separate storage of media components gave inferior confluence results compared to mixed storage regardless of storage temperature. Results at week 8 suggest that media and supplements should be stored at 4°C in order to preserve shelf-life for up to 8 weeks of storage or use. At 10 and 12 weeks, even storage at 4°C did not protect from a loss of culture confluence below acceptable levels (75%). The early (weeks 4 and 6) decrease in confluence in the 37°C groups does not appear to be due to changes in the glucose content or pH of the media, since these were relatively stable when confluence had fallen below acceptable levels or morphology was clearly abnormal. However, the fairly uniform reductions in confluence observed at week 8 and beyond may be due, at least in part, to the decreased glucose content and increased pH of the media which is observed at this point in the study.

The trend toward increasing mitochondrial metabolism over the course of the study as indicated by absorbance in the MTT assay, may be a sign that the cultures are nutrient deprived. Nutrient deprivation can refer to deficits in essential amino acids, growth factors, hormones, or other growth-promoting factors. A deficit in ATP, the energy currency of the cell, may stimulate the proliferation of mitochondria as a compensatory response to the lack of end product. Mitochondria perform oxidative phosphorylation, using NADH and FADH<sub>2</sub>, to produce ATP. Increased mitochondrial metabolism may be a reflection of greater numbers of mitochondria and/or increased activity in existing mitochondria. The abnormal cell morphology observed is compatible with nutrient deprivation because this stimulus can alter gene activity which in turn can alter morphology.

**In summary, it is recommended that cell culture media and supplements be stored mixed in solution, rather than in separate containers, at temperatures between 4°C and 22°C for 6 weeks of shelf-life and at 4°C for up to 8 weeks of shelf-life. It is recommended that media and supplements not be stored longer than 8 weeks because even storage at 4°C cannot maintain shelf-life beyond this point.**

## **MEDIA STORAGE CONDITIONS**

### **FIGURES**

#### **FIGURE LEGENDS:**

**D = Dulbecco's Modified Eagle's Medium (DMEM, basal media)**

**F = Fetal Bovine Serum**

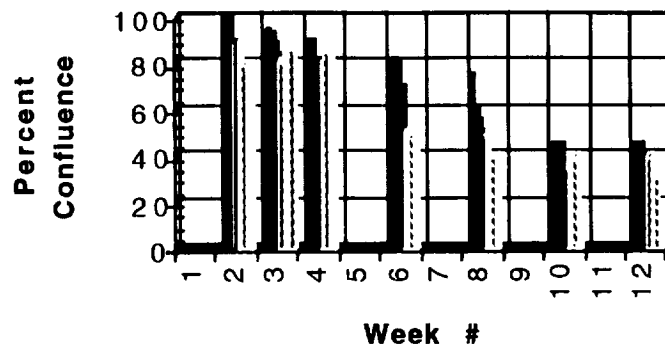
**G = Glutamine**

**GD = Glutamax-DMEM (contains a more stable form of glutamine)**



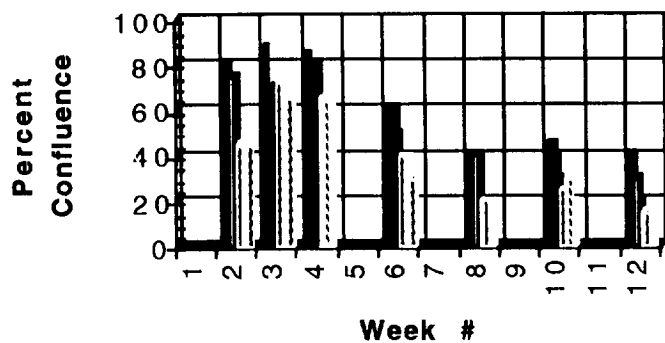
**Culture Confluence Fig. 1A**

Components stored mixed



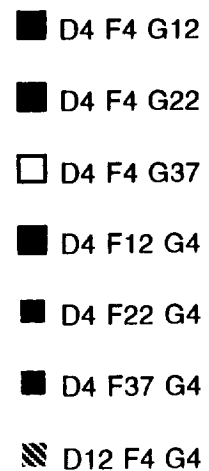
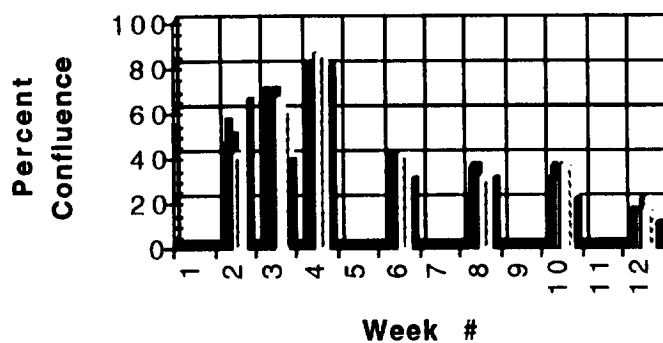
**Culture Confluence Fig. 1B**

Components stored separately



**Culture Confluence Fig. 1C**

Components stored separately







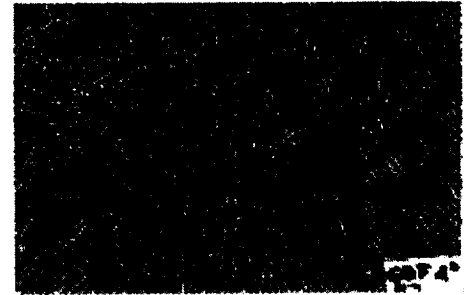
## Fig. 2 Week 3 of storage

DMEM + FBS + glutamine

G-DMEM + FBS + glutamine



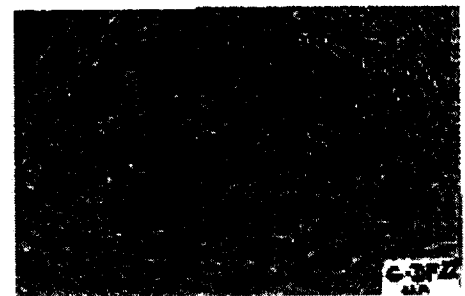
4°C



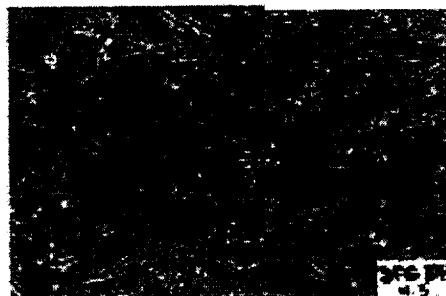
4°C



22°C



22°C



37°C



37°C



# Fig. 3 Week 3 of storage



DMEM 4°C  
FBS 4°C  
Glutamine 12°C



DMEM 4°C  
FBS 4°C  
Glutamine 37°C



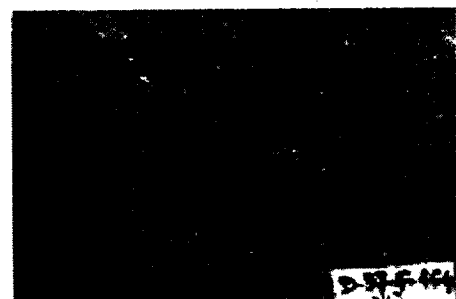
DMEM 4°C  
FBS 12°C  
Glutamine 4°C



DMEM 4°C  
FBS 37°C  
Glutamine 4°C



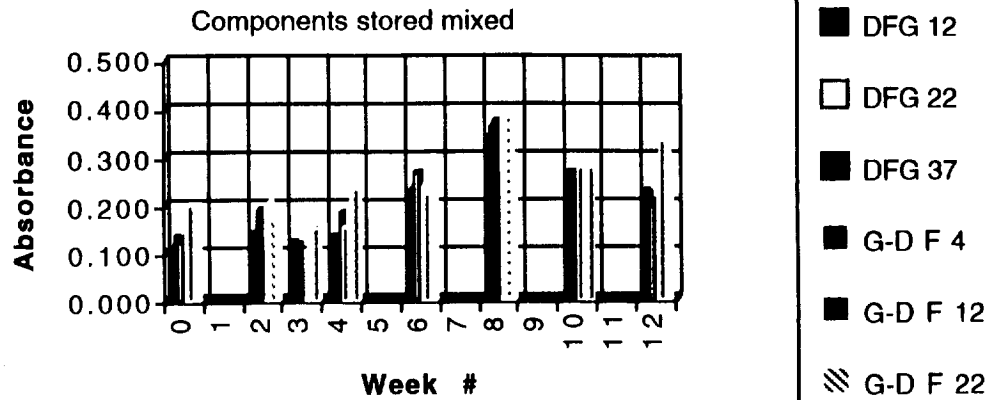
DMEM 12°C  
FBS 4°C  
Glutamine 4°C



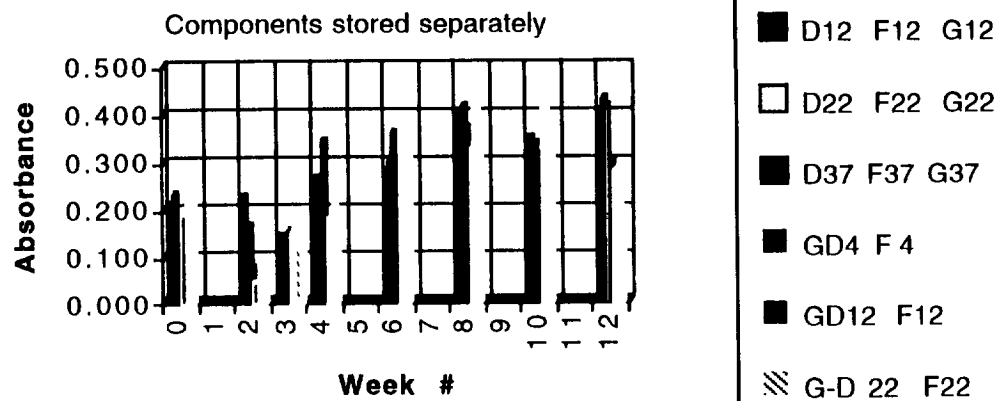
DMEM 37°C  
FBS 4°C  
Glutamine 4°C



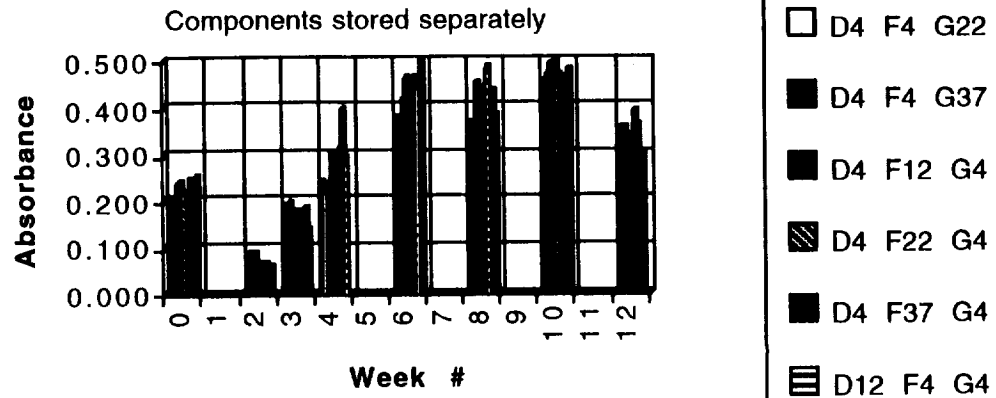
**MTT assay Fig.4A**



**MTT assay Fig. 4B**



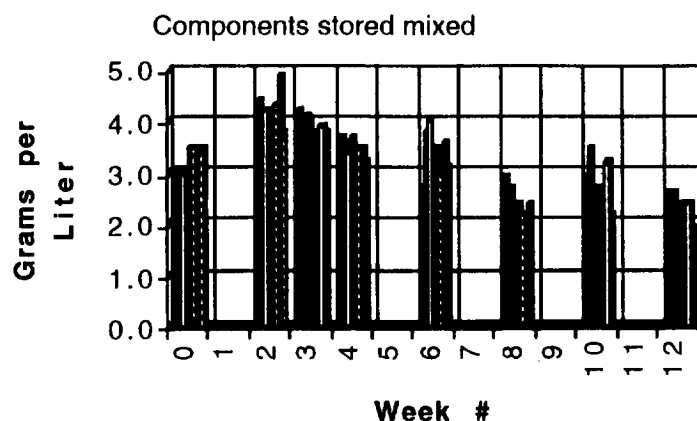
**MTT assay Fig. 4C**





### Glucose Content

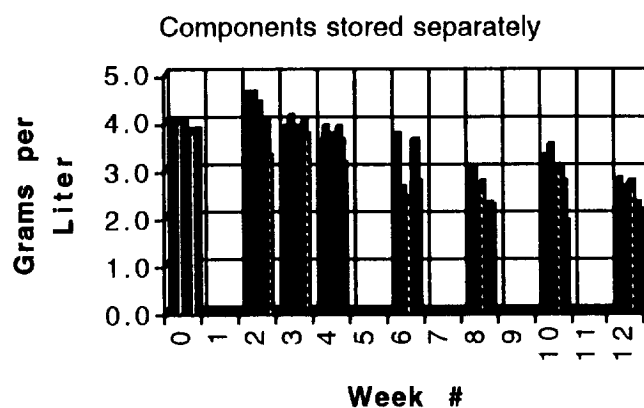
Fig. 5A



- DFG 4
- DFG 12
- DFG 22
- DFG 37
- ▨ G-D F 4
- ▨ G-D F 12
- G-D F 22

### Glucose Content

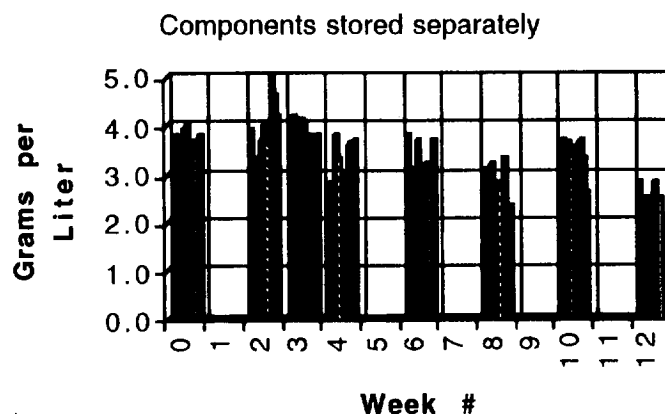
Fig. 5B



- D4 F4 G4
- D12 F12 G12
- D22 F22 G22
- D37 F37 G37
- ▨ GD4 F 4
- ▨ GD12 F12
- G-D 22 F22

### Glucose Content

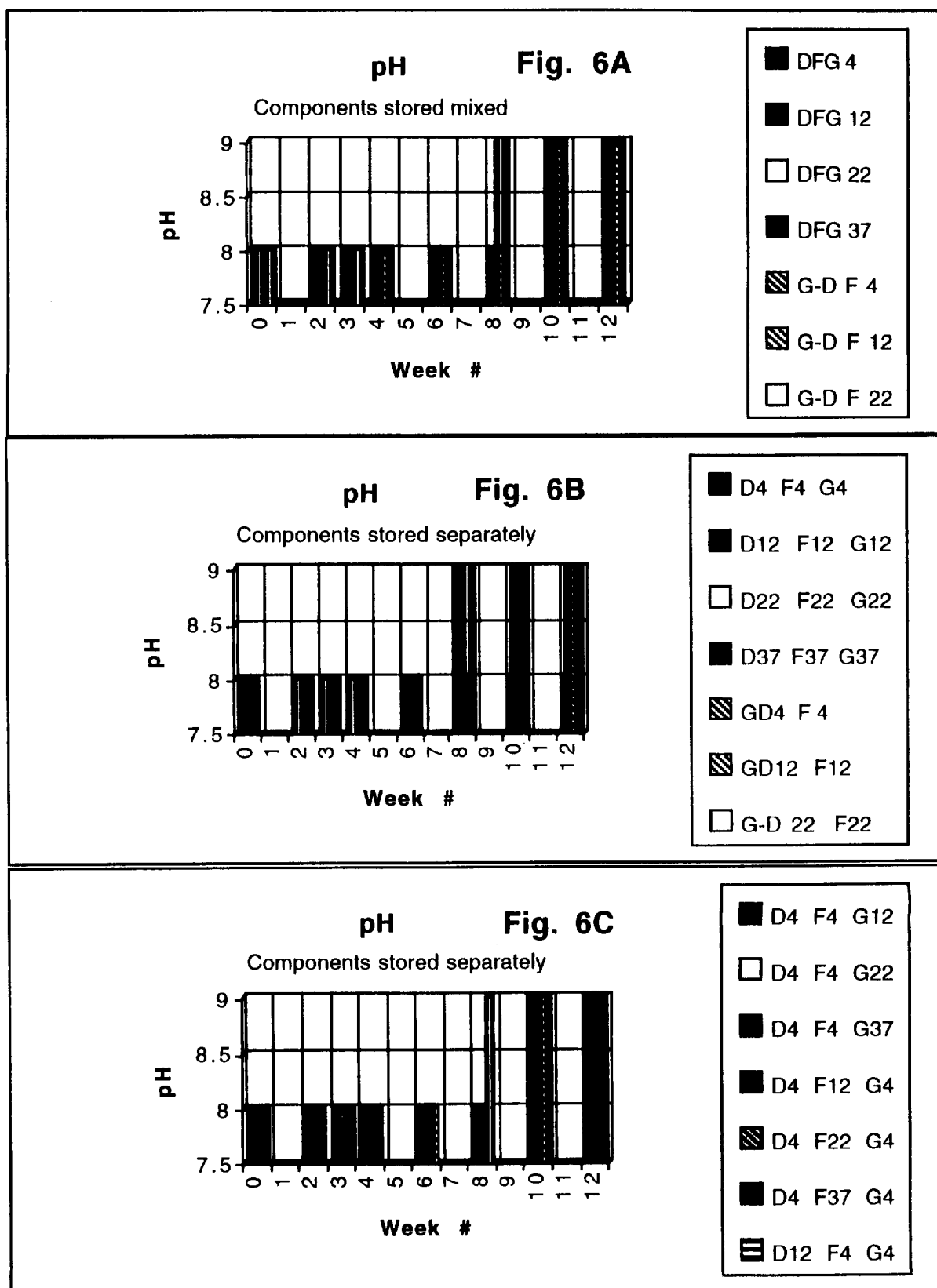
Fig. 5C



- D4 F4 G12
- D4 F4 G22
- D4 F4 G37
- ▨ D4 F12 G4
- D4 F22 G4
- ▨ D4 F37 G4
- D12 F4 G4









**FIXATIVE SOLUTION MANAGEMENT  
FINAL REPORT**



## **Fixative Solution Management Final Report**

### **1.0 PURPOSE**

The purpose of this final report is to document the experimental activities conducted and the results obtained, and to formulate a recommendation concerning temperature storage conditions required to support long-term fixative stability. The purpose of this study was to determine the long-term shelf life of commonly used fixative solutions stored in the dark at temperatures ranging from 4°C to 37°C. Chemical analysis was used to determine the stability of a series of fixatives at various concentrations in buffer as a function of storage duration and temperature. This study was performed by Martin Marietta Services at Ames Research Center.

### **2.0 BACKGROUND**

The Gravitational Biology Facility project is designing a suite of hardware for conducting long duration cell, developmental, and plant research on Space Station. Fixatives may have to be stored for up to 90 days on orbit prior to use. Chemical fixation is a standard means of preserving biological samples in ground-based laboratories for later histological or cytochemical analysis. Fixation is particularly important in space research; in order to eliminate the effects of reentry and landing on experimental results obtained in space, many life scientists prefer to preserve samples using on-orbit fixation prior to analysis in ground-based laboratories. However, very little information exists in the literature about the chemical integrity of fixative solutions when stored at other than the standard refrigeration temperature of 4°C, which is difficult to obtain within the power limitations encountered on Station. Therefore, this study was undertaken to better define the storage temperature requirements of commonly used fixative solutions. The need for this information arises from the limited electrical power and cold storage space available within the Cell Culture Unit of Space Station for long-term (up to 90 days) storage of fixative solutions.

High Performance Liquid Chromatography (HPLC) was used to determine the stability of a series of fixatives at various concentrations in buffer as a function of storage duration and temperature. Seven fixative solutions were tested at two week intervals for a period of 84 days. High and low concentrations of paraformaldehyde and glutaraldehyde, alone and in combination, were examined as well as a relatively new aldehyde-based fixative, Histochoice™ (Amresco; Solon, OH). Paraformaldehyde and glutaraldehyde are routinely used in earth laboratories, particularly in combination, for electron microscopy work, and have been frequently used on orbit to fix biological specimens. Histochoice™ is packaged "ready for use" and has the advantage of being relatively non-toxic and designed for use at room temperature. However, it is not recommended for use in electron microscopy (M. Behm of Amresco, Inc., personal communication).

### 3.0 MATERIALS AND METHODS

Paraformaldehyde and glutaraldehyde solutions were prepared from paraformaldehyde powder and 70% aqueous glutaraldehyde (Sigma; St. Louis, MO), respectively, in 0.2 M phosphate buffered saline (Sigma Immuno Chemicals; St. Louis, MO). Histochoice™ was purchased from Amresco, Inc. (Solon, OH). The intent was to test both a low and a high dose of each fixative in order to assess the effect of initial concentration on subsequent stability. The initial concentration of the fixative solutions, as determined by subsequent HPLC analysis, were:

- 1) Low Dose Paraformaldehyde = 3.5% (pH 7.2-7.4)
- 2) High Dose Paraformaldehyde = 15% (pH 7.2-7.4)
- 3) Low Dose Glutaraldehyde = 5% (pH 6.8-7.0)
- 4) High Dose Glutaraldehyde = 25% (pH 6.5-7.0)
- 5) Low Dose Paraformaldehyde = 3.5%/Glutaraldehyde = 4.5% (pH 7.2-7.4)
- 6) High Dose Paraformaldehyde = 10%/Glutaraldehyde = 20% (pH 7.2-7.4)
- 7) Histochoice™ (100%; pH 3-4)

Initial concentrations were determined from a 2 ml sample taken from each stock solution immediately after preparation. Stock solutions were then aliquoted into 40 ml Wheaton glass bottles with Teflon-faced silicon septa screw caps. The bottle caps were wrapped in parafilm and two bottles of each solution were placed in a 4°C refrigerator, 12°C water bath, 22°C water bath, and 37°C incubator.

Within several days of storage at 4°C and 12°C, a precipitation was visible in many of the bottles, particularly the 22% paraformaldehyde and Histochoice™ solutions. Therefore, additional groups were set up on day 28 to determine whether precipitation was due to polymerization of fixative molecules. Stock solutions of paraformaldehyde, glutaraldehyde, and Histochoice™ were sampled for baseline measurements and then filtered through a 1 micron filter to remove any nucleation sites for polymerization. The filtrates were aliquoted into glass bottles with septa-containing screw caps wrapped in parafilm, and two bottles of each were stored at 4°C and 37°C. HPLC analysis showed no significant effect of filtration on initial (day 0) fixative concentrations.

All bottles were sampled at two week intervals after being placed at storage temperature. Two samples (2 ml each) from each bottle were collected through the septum of the cap using a 22 Ga needle and a 3 ml syringe. Samples were collected into 2 ml microcentrifuge tubes and delivered to the Central Analytical Chemistry Laboratory at NASA Ames Research Center for HPLC analysis. Fixative samples were stored at 4°C, except for Histochoice™ which was stored at room temperature, for no longer than 24 hours prior to analysis.

The aldehydes in the fixative samples were derivatized to form colored compounds which absorb at 357 nanometers. The derivatives were separated on an HPLC column and the absorbance of the separated peak was recorded. The amount of aldehyde present was calculated by comparison to the absorbance of a standard of known aldehyde concentration. A decrease in absorbance indicates a decrease in the concentration of the fixative solution and implies a decrease in the stability of the fixative. Detailed protocols for the analysis of glutaraldehyde/paraformaldehyde and Histochoice™ can be found in Appendix A.

The results of the two samples taken from each bottle were averaged and the results of the two bottles for each fixative solution were averaged. The data are expressed as percent of initial concentration (day 0). The relative standard deviation (S.D. in figures) of the assay was between 3.3% and 3.8% of the aldehyde concentration.

#### 4.0 **RESULTS**

##### 4.1 **Low Dose Paraformaldehyde**

The initial concentration of the low dose paraformaldehyde solution was 3.5% and appeared to be relatively stable at all the temperatures tested (Figure 1A). Concentrations varied between 85% and 110% of initial throughout the 84 days of storage, regardless of storage temperature. The one outlier in the figure (day 14, 4°C) most likely reflects a sampling error.

Filtration appeared to have little or no effect on the stability of 3.5% paraformaldehyde at 4°C or 37°C (Figure 1B) where the pattern resembled that found in unfiltered samples (Figure 1A). The only exception were the samples stored at 37°C which appeared to fall to 80% of initial between day 70 and day 84, which may represent a sampling error. Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown).

##### 4.2 **High Dose Paraformaldehyde**

The initial concentration of the high dose paraformaldehyde solution was 15% and, similarly to the low dose paraformaldehyde, appeared to be relatively stable at all the temperatures tested (Figure 2A). Concentrations varied between 100% and 150% of initial throughout the 84 days of storage, regardless of storage temperature. In all groups the concentration appeared to gradually increase over time, at various rates depending on the storage temperature, and then plateau. This may have been caused by the relative insolubility of paraformaldehyde; as storage duration increased, more of the fixative went into solution and the concentration appeared to increase.

Filtration appeared to have little or no effect on the stability of 15% paraformaldehyde at 4°C or 37°C (Figure 2B) where the pattern resembled that found in unfiltered samples (Figure 2A). Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown).

##### 4.3 **Low Dose Glutaraldehyde**

The initial concentration of the low dose glutaraldehyde solution was 5% and appeared to be least stable when stored at 37°C; the concentration fell to 50% of initial after 28 days where it remained through day 84 (Figure 3A). Storage at 4°C, 12°C, or 22°C produced variations in the concentration between 110% and 80% of initial throughout the 84 days of storage.

Filtration appeared to have little or no effect on the stability of 5% glutaraldehyde at 4°C or 37°C (Figure 3B) where the pattern of degradation resembled that found in unfiltered samples (Figure 3A). Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown). Filtered glutaraldehyde stored at 37°C turned slightly yellow during the last two weeks of the test period (not shown).

#### 4.4 High Dose Glutaraldehyde

The initial concentration of the high dose glutaraldehyde solution was 25% and appeared to be most sensitive to degradation when stored at 22°C or 37°C (Figure 4A). Storage at 22°C produced a decrease to 80% of initial concentration by day 28 and only a slight decrease thereafter. Storage at 37°C produced a decrease to 45% of initial by day 28 and remained between 50% and 60% thereafter. Storage at 4°C or 12°C produced variations in the concentration between 110% and 90% of initial throughout the 84 days of storage.

Filtration appeared to have little effect on the stability of 25% glutaraldehyde at 4°C or 37°C (Figure 4B) where the pattern of degradation resembled that found in unfiltered samples (Figure 4A). Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown). Both filtered and unfiltered glutaraldehyde stored at 37°C turned yellow, the unfiltered earlier and more intensely than the filtered, during the last 4-6 weeks of the test period (not shown).

#### 4.5 Low Dose Paraformaldehyde/Gutaraldehyde

The initial concentration of the low dose paraformaldehyde/gutaraldehyde solution was 3.5% paraformaldehyde and 4.5% glutaraldehyde. Both fixatives showed a temperature-dependent degradation over time, with glutaraldehyde faring worse at 22°C and 37°C compared to paraformaldehyde (Figure 5A). Storage at 22°C produced a decrease to 50% of initial for paraformaldehyde and 40% of initial for glutaraldehyde by day 84; the sharpest decrease in concentrations occurred between day 0 and day 14. Storage at 37°C produced a decrease to 45% of initial for paraformaldehyde and 25% of initial for glutaraldehyde by day 84; again, the sharpest decrease in concentrations occurred between day 0 and day 14. The pattern of stability at 4°C and 12°C appeared similar for the two fixatives.

Filtration appeared to have a small positive effect on the stability of 3.5% paraformaldehyde and 4.5% glutaraldehyde at 4°C and 37°C (Figure 5B); the concentrations were slightly higher than those of the unfiltered solutions stored at the same temperature (Figure 5A). Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown).

#### 4.6 High Dose Paraformaldehyde/Gutaraldehyde

The initial concentration of the high dose paraformaldehyde/gutaraldehyde solution was 10% paraformaldehyde and 20% glutaraldehyde. Both fixatives showed a temperature-dependent degradation over time, with little difference between the two fixatives (Figure 6A). Storage at 22°C produced a decrease to 35% of initial for paraformaldehyde and 45% of initial for glutaraldehyde by day 84; the sharpest decrease was between day 14 and day 28 for paraformaldehyde and between day 0 and day 28 for glutaraldehyde. Storage at 37°C produced a decrease to 30% of initial for paraformaldehyde and 35% of initial for glutaraldehyde by day 84; the sharpest decrease in concentration occurred between day 0 and day 14 for both fixatives. The pattern of stability of the two fixatives at 4°C and 12°C appeared similar until between day 56 and day 68, when paraformaldehyde fell below the glutaraldehyde curve.



Filtration appeared to have a small positive effect on the stability of 10% paraformaldehyde and 20% glutaraldehyde at 4°C and 37°C (Figure 6B); the concentrations were slightly higher than the unfiltered solutions stored at the same temperature (Figure 6A). Filtration appeared to slightly decrease the amount of precipitation at 4°C and 37°C (not shown). Both filtered and unfiltered paraformaldehyde/glutaraldehyde stored at 37°C turned yellow, the unfiltered earlier and more intensely than the filtered, during the last 4-6 weeks of the test period (not shown).

#### 4.7 Histochoice™

Histochoice™ appeared to be as temperature stable as paraformaldehyde. The initial concentration (100%) remained intact through 84 days of storage at temperatures ranging from 4°C to 37°C (Figure 7A).

Filtration appeared to have little or no effect on either the stability of Histochoice™ at 4°C or 37°C (Figure 7B) or the precipitation observed at 4°C (not shown). Precipitation at 37°C was not observed before or after filtration.

#### 5.0 SUMMARY AND CONCLUSIONS

This study was performed to determine the temperature storage requirements of several common fixative solutions likely to be used for preservation of biological specimens on Space Station. This study also addressed the influence of initial concentration and filtration on subsequent stability of these fixatives.

Recommended storage temperatures of fixatives to be used on Space Station depends on the working concentration required to adequately fix the specimen of interest, the duration of the experiment, the particular fixative to be used, and the potential effects of breakdown products. It should be noted that this study did not address the ability of the fixatives tested to adequately fix cells or tissues. Therefore, the recommendations formulated here are valid only within the scope of this study which focused on chemical analysis of fixative stability.

The resulting ranking of fixative stability, as determined by HPLC, at storage temperatures between 4°C and 37°C over an 84 day period was found to be:

paraformaldehyde = Histochoice™ > glutaraldehyde >  
paraformaldehyde/glutaraldehyde

There appeared to be little or no effect of initial concentration or filtration on subsequent rate of degradation of the fixative solutions. Storage temperatures of 4°C and 12°C maintained fixative stability better than higher temperatures, particularly in the case of glutaraldehyde and paraformaldehyde/glutaraldehyde solutions, but were associated with some degree of precipitation of all the fixatives tested. Furthermore, given the electrical power constraints on Space Station, these temperatures are difficult to achieve and maintain, particularly when cold storage space must be located near cell/tissue culture incubation areas within the BioCulture System or other life science research hardware.

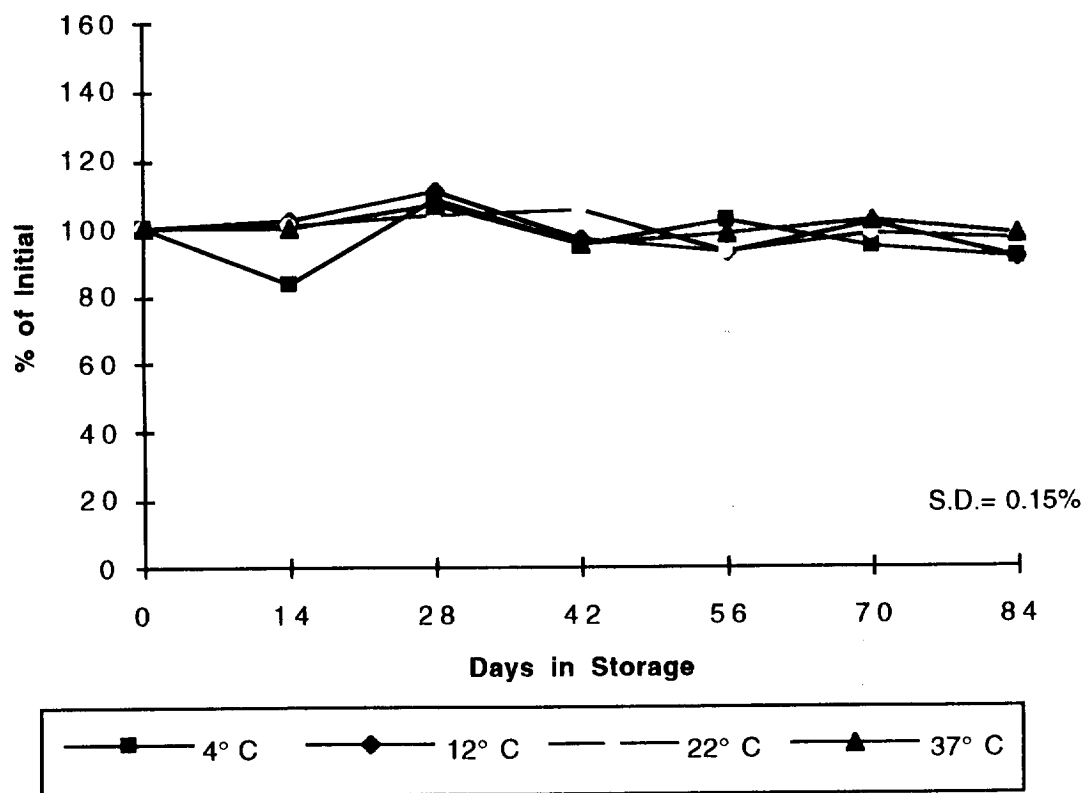
Storage at 37°C is acceptable for low and high dose paraformaldehyde and Histochoice™ solutions, which showed little or no degradation at storage temperatures ranging between 4°C and 37°C for a period of 84 days. Furthermore, if the assumption is made that a working concentration of at least 2% aldehyde is required to adequately fix most biological specimens, then a storage temperature of 37°C is sufficient to maintain the integrity of all the fixative types tested, with the exception of the low dose paraformaldehyde (3.5%)/glutaraldehyde (4.5%) solution, which lost between 60% and 80% of initial concentration when stored at 37°C. Initial concentration of this fixative solution would have to be sufficient to compensate for the degradation observed, primarily during the first 14 days, at 37°C. The same is true for low and high dose glutaraldehyde solution, which lost approximately 50% of initial concentration during the first 28 days of storage at 37°C. Initial concentration of this fixative solution would also have to be sufficient to compensate for degradation observed at 37°C. Ideally, a storage temperature of 22°C would be preferable for glutaraldehyde and paraformaldehyde/glutaraldehyde solutions because it produced less degradation than storage at 37°C.

## **FIXATIVE SOLUTION MANAGEMENT FIGURES**



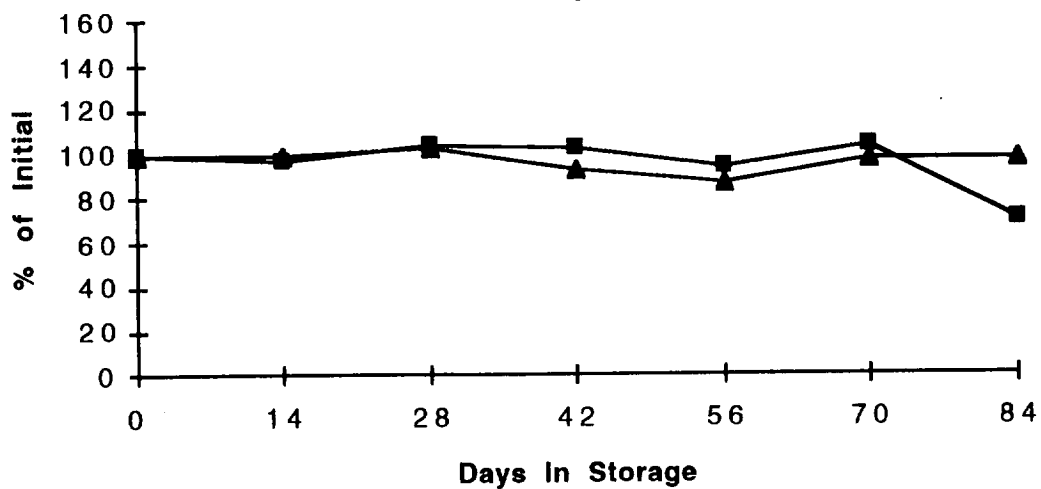
# LOW DOSE PARAFORMALDEHYDE

Figure 1A



# Filtered LOW DOSE PARAFORMALDEHYDE

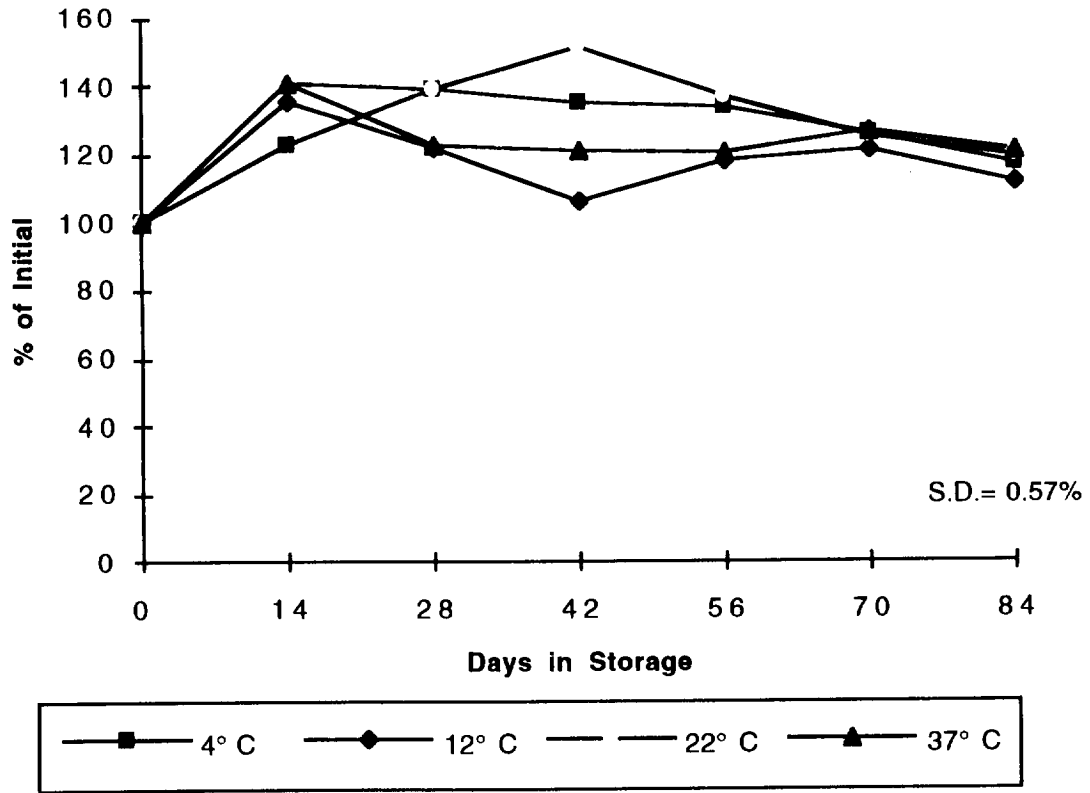
Figure 1B





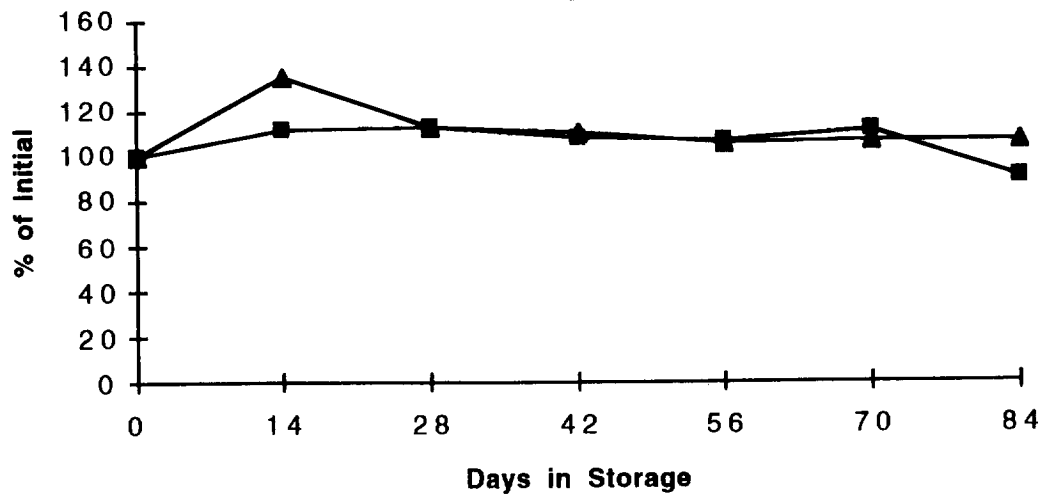
### HIGH DOSE PARAFORMALDEHYDE

Figure 2A



### Filtered HIGH DOSE PARAFORMALDEHYDE

Figure 2B

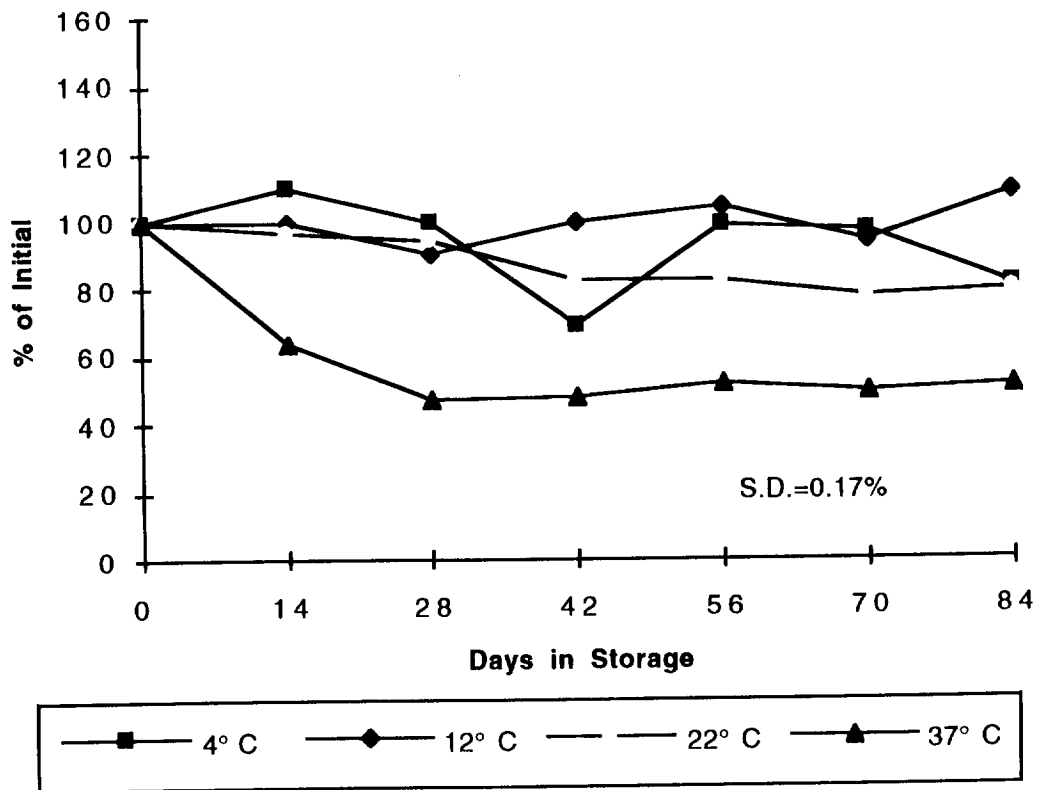






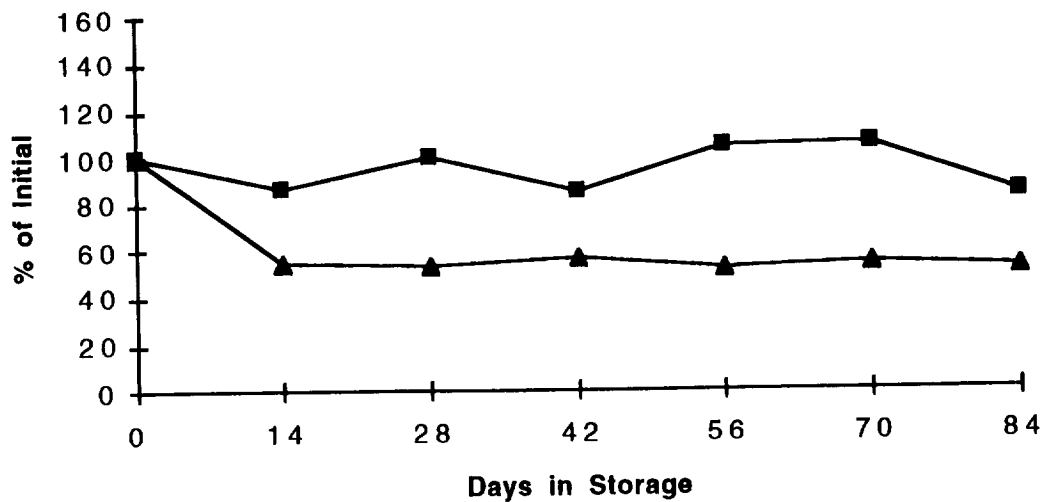
# LOW DOSE GLUTARALDEHYDE

Figure 3A



## Filtered LOW DOSE GLUTARALDEHYDE

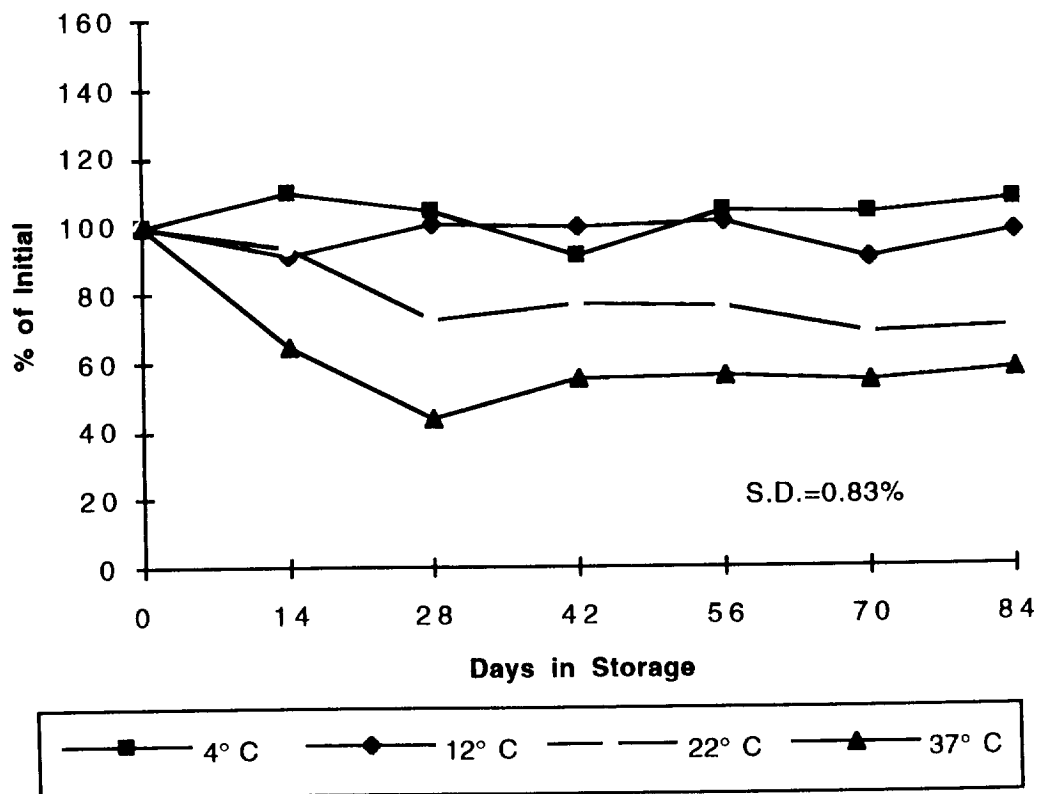
Figure 3B





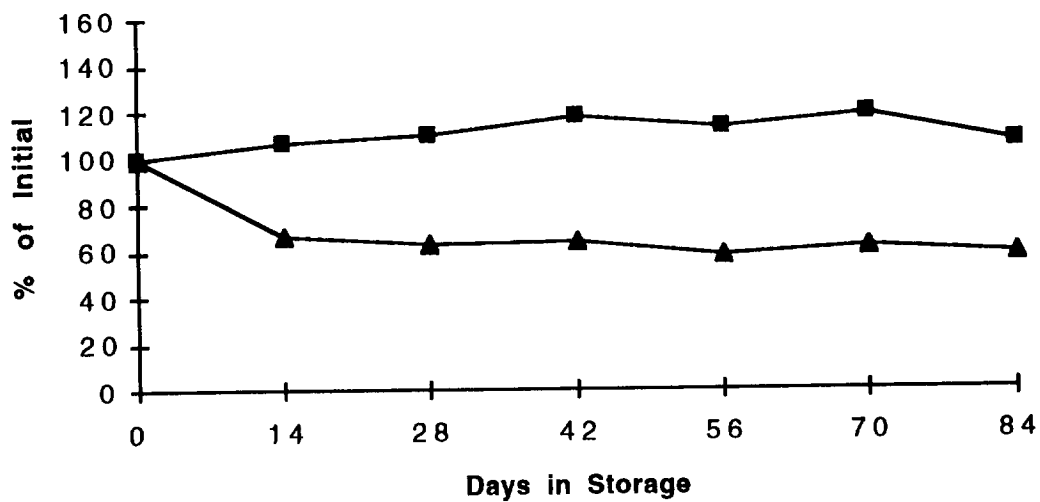
# **HIGH DOSE GLUTARALDEHYDE**

**Figure 4A**



# **Filtered HIGH DOSE GLUTARALDEHYDE**

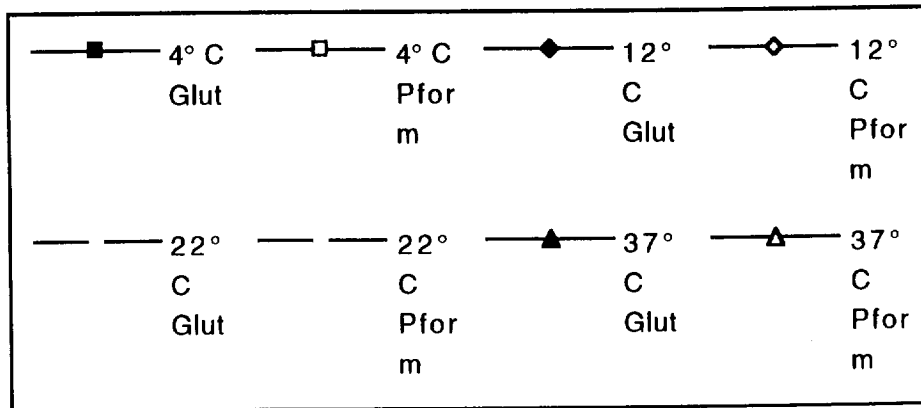
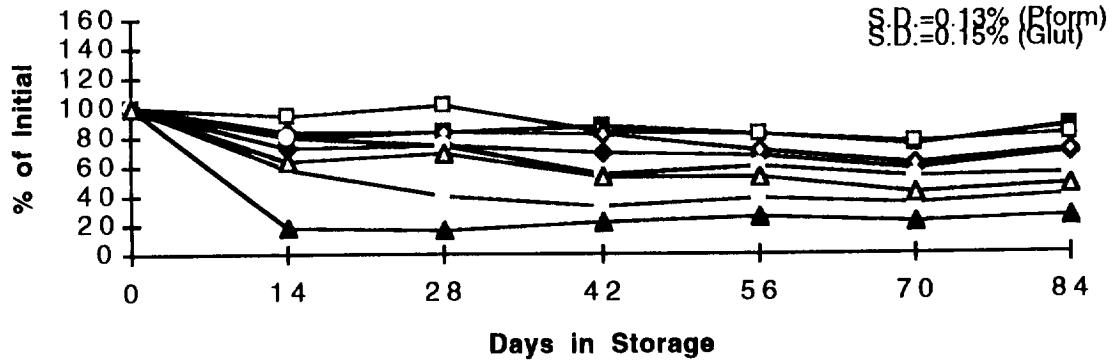
**Figure 4B**





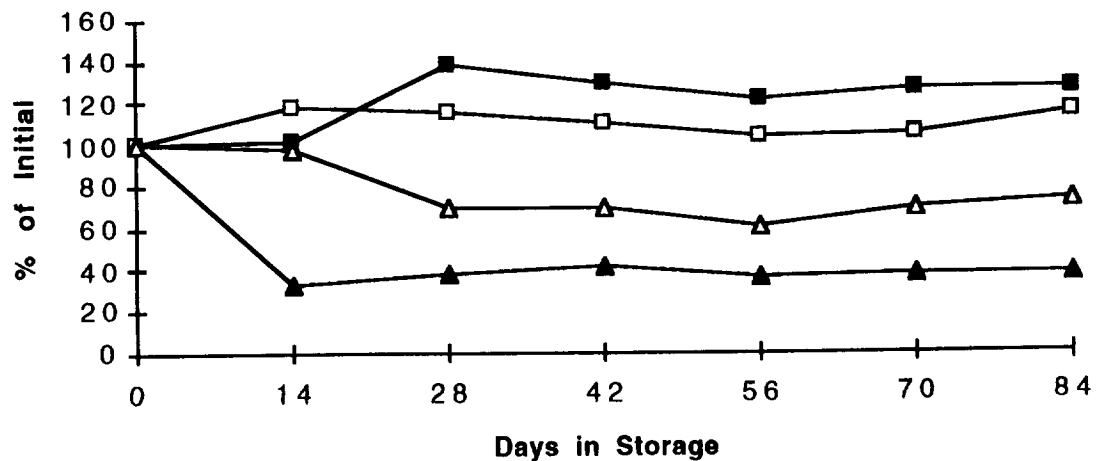
# LOW DOSE PARAFORMALDEHYDE / GLUTARALDEHYDE

Figure 5A



# LOW DOSE PARAFORMALDEHYDE / GLUTARALDEHYDE

Figure 5B

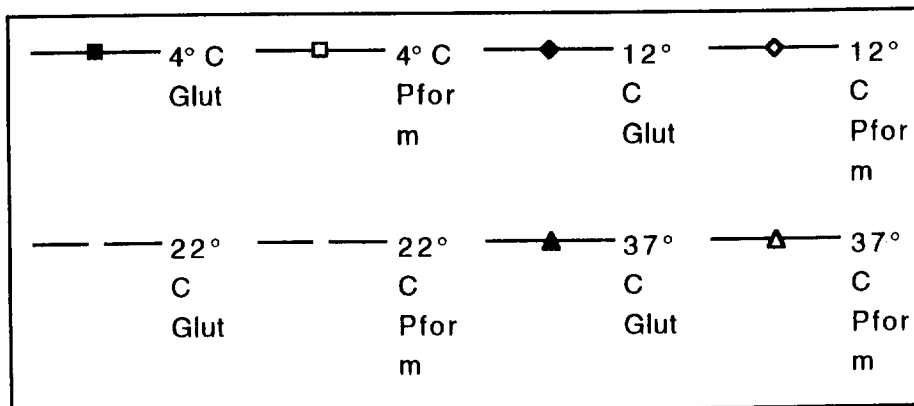
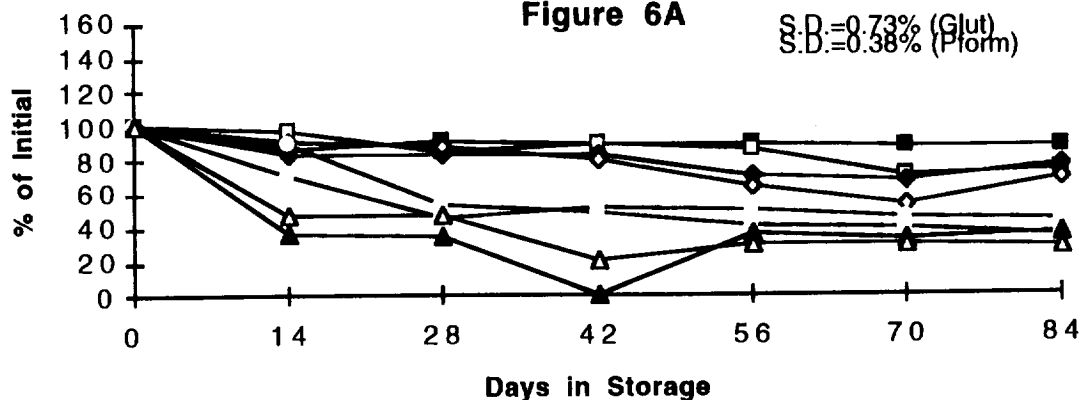




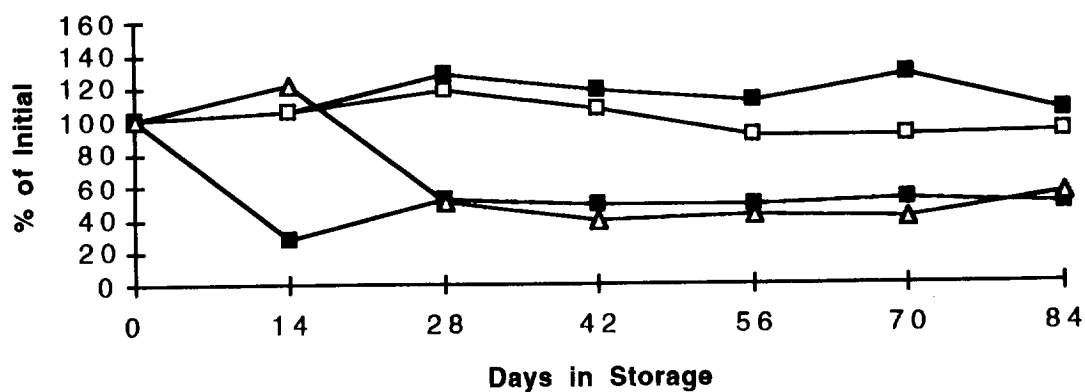
# HIGH DOSE PARAFORMALDEHYDE / GLUTARALDEHYDE

Figure 6A

S.D.=0.73% (Glut)  
S.D.=0.38% (Pform)



## Filtered HIGH DOSE PARAFORMALDEHYDE / GLUTARALDEHYDE Figure 6B

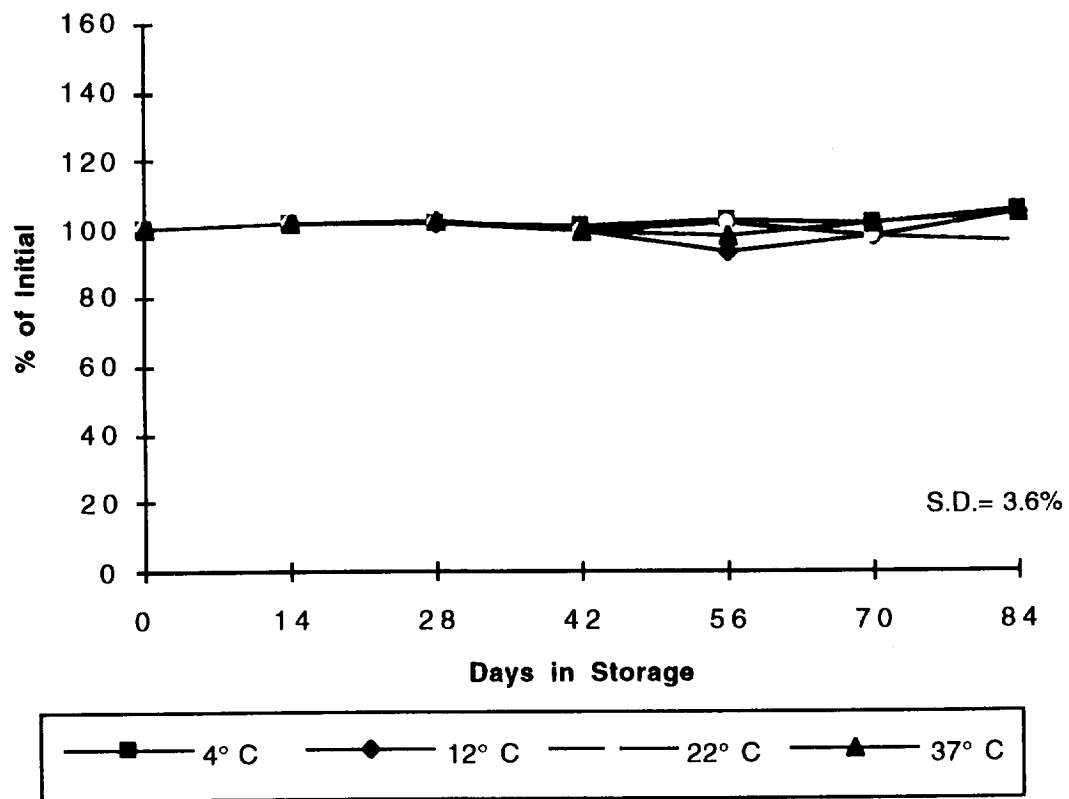






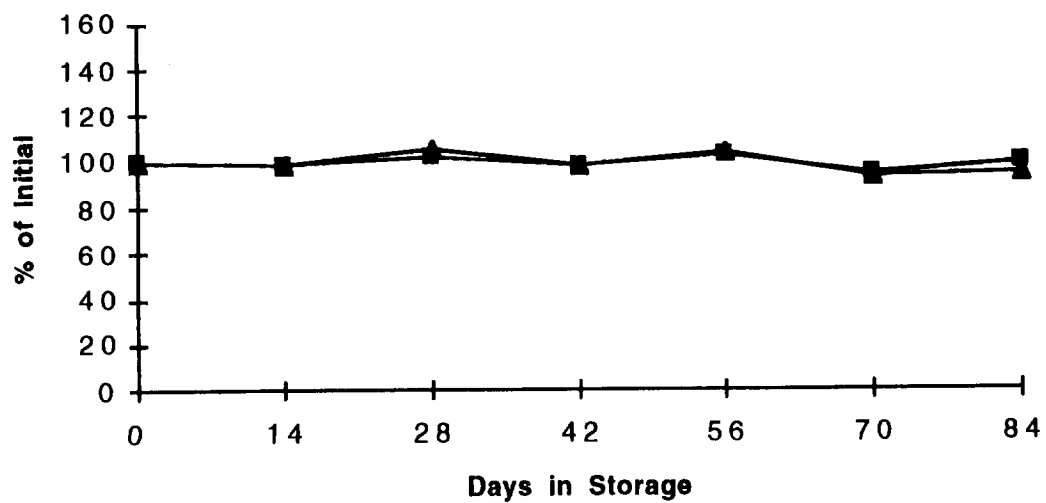
## HISTOCHOICE™

Figure 7A



## Filtered HISTOCHOICE™

Figure 7B





## **APPENDIX A**

### **ANALYTICAL OPERATING PROCEDURES FOR THE DETERMINATION OF ALDEHYDES AND HISTOCHOICE™**



**Analytical Operating Procedure**  
**Aldehydes via DNPH Derivatives**  
**Written By: Warren Belisle**  
**Belisle**

**Document # ACL 10031**  
**Revision Date: 7/18/94**  
**Approved By: Warren**

1. **PURPOSE:**

The purpose of this procedure is to describe the methodology to measure the quantities of select aldehydes in solution.

Note: A 100 % paraformaldehyde solution refers to a 37% solution of formaldehyde in water. Alcohols such as methanol may be added to the solution to inhibit polymerization.

2. **SCOPE:**

This procedure includes the quantitation of formaldehyde and glutaraldehyde in solution. Other aldehydes can be measured but the chromatograph and possible interferences must first be investigated.

Aldehydes react with dinitrophenylhydrazines in an acidic medium to form a dinitrophenylhydrazone which absorbs at 357 nanometers. The hydrazones which are formed are separated on an HPLC column and the absorbance of the separated peak is recorded. The amount of aldehyde present is calculated by comparison to the absorbance of a standard of known aldehyde concentration.

3. **STANDARDS AND SOLUTIONS:**

3.1 **DNPH Reagent**

Dinitrophenylhydrazine	0.025 g
6N HCl q.s. ad	10.0 ml

Outdate: 6 months

3.2 **6N HCl**

HCl Concentrated	50 ml
Deionized H <sub>2</sub> O	50 ml

Caution: Always add the acid to the water. Perform this operation in the fume hood.

Outdate: 6 months



**Analytical Operating Procedure**  
**Aldehydes via DNPH Derivatives**

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**3.3 Stock Aldehyde Standard**

Formaldehyde (37%)	0.4 ml
Glutaraldehyde (50%)	0.4 ml
Deionized H <sub>2</sub> O q.s.ad	100.0 ml
Formaldehyde = 1480 ppm	
Glutaraldehyde = 2000 ppm	
Store in the refrigerator	Outdate: 3 months

**3.4 Aldehyde Working Standard**

Stock Aldehyde Standard	3.0 ml
Deionized H <sub>2</sub> O q.s. ad	10.0 ml

Formaldehyde = 444 ppm  
Glutaraldehyde = 600 ppm

Store in the refrigerator and protect from light.  
Outdate 2 weeks

**3.5 Acetonitrile-Water 50%**

Acetonitrile	100 ml
Deionized Water	100 ml

Outdate 3 months

**4.0 SAMPLE PREPARATION**

**4.1 4% Glutaraldehyde - (Prepare in duplicate)**

Sample	0.15 ml
Deionized H <sub>2</sub> O q.s. ad	10.0 ml

Store in the refrigerator and protect from light.  
Outdate: 2 days  
Factor = 4.00

**4.2 22% Glutaraldehyde - (Prepare in duplicate)**

Sample	0.15 ml
Deionized H <sub>2</sub> O q.s. ad	50.0 ml

Store in the refrigerator and protect from light.  
Outdate: 2 days  
Factor = 20.00



Analytical Operating Procedure  
Aldehydes via DNPH Derivatives

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4.3 **4% Paraformaldehyde** - (Prepare in duplicate)

Sample	0.1 ml
Deionized H <sub>2</sub> O q.s. ad	10.0 ml

Store in the refrigerator and protect from light.

Outdate: 2 days

Factor = 4.44

4.4 **22% Paraformaldehyde** - (Prepare in duplicate)

Sample	0.1 ml
Deionized H <sub>2</sub> O q.s. ad	50.0 ml

Store in the refrigerator and protect from light.

Outdate: 2 days

Factor = 22.2

4.5 **4% Formaldehyde, 4% Glutaraldehyde** - (Prepare in duplicate)

Sample	0.1 ml
Deionized H <sub>2</sub> O q.s. ad	10.0 ml

Store in the refrigerator and protect from light.

Outdate: 2 days

Factor = 4.44 Formaldehyde and 6.00 for Glutaraldehyde

4.6 **11% Formaldehyde, 11% Glutaraldehyde** - (Prepare in duplicate)

Sample	0.1 ml
Deionized H <sub>2</sub> O q.s. ad	25.0 ml

Store in the refrigerator and protect from light.

Outdate: 2 days

Factor = 11.1 for Formaldehyde and 15.0 for Glutaraldehyde

5.0 **PROCEDURE**

5.1 **HPLC Parameters**

The following parameters are for the Shimadzu Model LC-7A HPLC system equipped with the SPD-M6A photo diode array detector.



Analytical Operating Procedure  
Aldehydes via DNPH Derivatives

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Column: C-18 silica 5 micron 25 cm column  
Solvent A: Water  
Solvent B: Acetonitrile  
Flow Rate: 1 ml per minute  
% B: 70 % isocratic  
Wavelength: 357 nm  
Run Time: 12 minutes  
Injection Size: 20 microliters  
Lamp: Deuterium

## 5.2 Analysis

- 5.2.1 Prepare duplicate hydrozone derivatives of Aldehyde Working Standard and duplicate sample dilutions as follows:

Acetonitrile-Water 50%	10.0 ml
Diluted sample or Working Std	0.1 ml
DNPH Reagent	0.3 ml

Allow the solution to stand for 30 minutes at room temperature which enables the reaction to go to completion.

Store at room temperature. Outdate 1 week

- 5.2.2 Prepare duplicate blanks by substituting deionized water for the sample or standard in section 5.2.1 above.
- 5.2.3 Fill the solvent A and solvent B containers, de-gas the solvents, turn on the pumps, turn on the detector, and press the D2 button to ignite the deuterium lamp.
- 5.2.4 Once the system has equilibrated, inject the blank preparation followed by the two standard preparations.
- 5.2.5 Inject each sample preparation.

## 5.3 Quantitation

% Formaldehyde =  $\text{Area (sample)} / \text{Area (Std)} * 444 \text{ ppm} * \text{dilution factor} / 10,000$

% Glutaraldehyde =  $\text{Area (sample)} / \text{Area (Std)} * 600 \text{ ppm} * \text{dilution factor} / 10,000$

Report the average of the duplicate sample results.



**Analytical Operating Procedure**  
**Operation of Shimadzu HPLC**  
**Written By: Warren Belisle**

**Document # ACL 10032**  
**Revision Date: 5/13/94**  
**Approved By: Warren Belisle**

1. **PURPOSE:**

The purpose of this procedure is summarize the operation of the Shimadzu high performance liquid chromatograph (HPLC). Refer to the instrument manual for a more in-depth description of the operation of the instrument.

2. **SCOPE:**

This procedure applies only to the Shimadzu HPLC which consists of dual pumps model LC-7A controlled by the system controller model SCL-6B. The detection system consists of a photo diode array detector model SPD-M6A. The signal from the detector along with the detector controller interface cable is attached to and controlled from a personal computer. The injection system is a manual Rheodyne injector.

3. **SET-UP PROCEDURE:**

3.1 **Column Installation**

- 3.1.1 Open the column oven door and disconnect the guard column and the analytical column. Cap the ends of the guard and analytical to prevent solvent evaporation.
- 3.1.2 Attach the new guard column to the exit line from the pumps. Do not over-tighten the fitting. Tighten to finger tight and additional 1/2 turn is usually sufficient to prevent leaks.
- 3.1.3 Observe the arrow on the analytical column for the direction of installation. Attach the analytical column to the exit of the guard column with a short length of 1/16th inch stainless tubing. Avoid over-tightening the fittings.
- 3.1.4 Attach the tubing going to the detector to the exit of the analytical column.

3.2 **Initial Start-up Procedure**

- 3.2.1 Turn on the system controller if it is not already on.
- 3.2.2 Turn on the column oven and set to 40 degrees C if it is not already on.
- 3.2.3 Turn on the power to the photo diode detector.
- 3.2.4 Turn on the power to the two pumps.
- 3.2.5 Turn on the computer. The photo diode array detector software will automatically load and the Utility menu will be displayed.
- 3.2.6 If a new data directory is required for the analysis, highlight SPDM6A Utilities followed by highlighting Prepare New Data Directory. Enter the name for the new data sub-directory and the appropriate files will be copied to the directory.
- 3.2.7 Highlight the Manual Operation option and press enter





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**Operation of Shimadzu HPLC**

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- 3.2.8 Highlight the desired data directory and press enter. The main screen for the detector will be displayed.

**3.3 Solvent Delivery System**

**3.3.1 Solvent Preparation**

- 3.3.1.1 Filter the solvents through a .45 micron Nylon 66 membranes to remove any particulates in addition to removing any dissolved gases.
- 3.3.1.2 Carefully transfer the filtered solvent into the solvent bottle to minimize the introduction of air into the solvent.
- 3.3.1.3 Empty the waste container into the hazardous waste storage containers.

**3.3.2 System Controller**

- 3.3.2.1 The system controller is used to control the flow of eluents through the pumps. It controls the flow and any gradient profile required during an analysis.
- 3.3.2.2 The system controller contains five main screens. The "Param" screen allows the operator to manually change the solvent composition and flow rate. The "Pump Ctrl" screen provides a graphical representation of the solvent gradient profile for the specified analysis. The "Time Prog" screen allows one to build a time program which will change the solvent composition at various times during the analytical run. The "Monitor" screen displays the actual flows and pressures for the pumping system. The "A.Inj" screen is for the auto-injector accessory which is not used at this time. The majority of the time one works from the "Param" screen.
- 3.3.2.3 You must be in the "Param" screen to turn on and turn off the pumps from the system controller.
- 3.3.2.4 You must be in the "Time Prog" screen to abort a gradient run.
- 3.3.2.5 There are 10 files where pump parameters can be stored. The files are numbered 0 - 9 and can be accessed from the "Param" screen on the system controller. Call up the file for the analysis of interest.
- 3.3.2.6 From the "Param" screen, start the pumps by pressing "Start" on the system controller's panel.

**3.3.3 Priming the Pumps**

- 3.3.3.1 Once the Start button is pressed on the system controller, the preset solvent flow should begin. The pressure should also begin to increase and stabilize.
- 3.3.3.2 If the pressure does not increase or does not stabilize, it is necessary to prime the pumps.
- 3.3.3.3 To prime one of the pumps, connect the end of the plastic priming tubing to the bypass valve tubing on the front of the pump. Attach the other end of the tubing to a syringe and open the valve.



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**Operation of Shimadzu HPLC**

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- 3.3.3.4 Withdraw some of the solvent from the pump using the syringe and then close the valve.
- 3.3.3.5 Repeat this operation on the other pump if necessary. The pressure on the system should stabilize at this time.

**3.4 Photo Diode Array Detector**

- 3.4.1 The signal from the photo diode array detector is monitored from the computer. The sample chromatograms and peak integration parameters are stored in the computer. Section 3.2.5 of this procedure describes the steps necessary to monitor and control the output of the chromatogram.
- 3.4.2 It may be necessary to perform a wavelength calibration when a new lamp is installed in the detector or at other times when un-explained background data are obtained. To calibrate the wavelength, highlight "Wavelength Calibration" from the main menu (press F6 if necessary). Each step of the calibration will halt and wait for F1 command to continue. Continue to give the F1 (continue) command until a numerical count down is observed on the top of the screen. At this time press the D2 button on the detector and wait for the deuterium lamp to light as indicated by the green light not blinking any more. At this time, press F1 continue.
- 3.4.3 There are two Real Time Analysis Modes available. One is a two channel wavelength range monitoring and the other is a 3D wavelength scan vs. absorbance vs. time graphical representation of the chromatogram. Select a mode of preference for the given application.
- 3.4.4 From the Real Time Analysis Mode screen the following parameters are entered by pressing F1 (Set1):
  - 3.4.4.1 Time Range (min/FS): sets the number of minutes for full scale of the X axis.
  - 3.4.4.2 Abs Range (AU/FS): sets the full scale absorbance range for the graphical output of the chromatogram.
  - 3.4.4.3 Lamp Status: sets either the tungsten or deuterium lamp.
  - 3.4.4.4 CH1 - Wavelength (nm): used in the multi-chromatogram mode to set the wavelength in nanometers for the first output channel.
  - 3.4.4.5 CH2 - Wavelength (nm): same as above except for the second output channel.
  - 3.4.4.6 Wavelength Range (nm): used in the 3D chromatogram mode to set the desired wavelength range in nanometers for the chromatogram.
  - 3.4.4.7 Time Constant (s): Designates the acquisition rate.
  - 3.4.4.8 Zero % [ CH1/ CH2/ Ratio]: used in the multi-chromatogram mode to set the baseline position on the screen for channel 1, channel 2 and the ratio of the two channels.
  - 3.4.4.9 Mem Start Time (min): Sets the raw data acquisition starting time.



- 3.4.4.10 File Name (raw data): Designates the file name where the raw data is stored. **Note:** If a new name is not assigned the previous file will be written over if the run is started.

#### **4.0 RUNNING A CHROMATOGRAM**

- 4.1 Once the pressure on the solvent delivery pumps has stabilized and the detector has equilibrated, a sample, blank or standard may be injected.
- 4.2 Change the File Name in the SET 1 parameters to reflect the sample to be injected. Enter this information into the sample log.
- 4.3 The sample introduction is made with a Rheodyne injection valve. A fixed volume injection loop is used to control the sample size being injected. Typically a 20 microliter injection loop will be used.
- 4.4 Use the sample syringe equipped with the square end needle to fill the sample injection loop. Insure that the injection switch arm is on the lower (counter clockwise ) position. Inject about 1 ml of sample to wash any of the previous sample from the system.
- 4.4 Move the injection handle up (clockwise) to the inject position. The run automatically begins when the handle is in the inject position.

#### **5.0 STANDARD CALIBRATION**

##### **5.1 Standard Chromatogram**

The calibration procedure takes place in a post-run mode after the standard chromatographs for all standards have been run and the files saved to disk.

##### **5.2 Calibration Procedure**

- 5.2.1 After the standard chromatograms have been run, press F6 to enter the Menu screen.
- 5.2.2 Highlight the Quantitation option and press enter to go to the quantitation screen.
- 5.2.3 Press F1 to set the parameters for the standard file.
- 5.2.4 Enter the file name for the first standard.
- 5.2.5 Change the "Time Program File" to the name "Work"
- 5.2.6 Leave this window by pressing the End key.
- 5.2.7 Plot the chromatogram by pressing the F2 key
- 5.2.8 Press F3 to set the parameters for the quantitation.



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- 5.2.9 Change the "I.D. File" name to "Dummy"
- 5.2.10 Press F4 to perform the peak identification and integration sequence. The left and right arrows can be used to identify the start and stop of a peak if the established parameters were not properly set to do so.
- 5.2.11 Press F9 to save the peak data.
- 5.2.12 Press F8 to enter the quantitation screen and create a new calibration I.D. File for this application.
- 5.2.13 Method 2: Absolute Calibration Curve or Method 3: Internal Standard are the methods normally used. Select one of these methods.
- 5.2.14 Indicate either a 1 or 2 point standard curve.
- 5.2.15 Enter the Time Window parameter instead of the Time Band
- 5.2.16 Enter 10% for the normal time window
- 5.2.17 Press Enter and highlight the file name for the peak area data for this standard
- 5.2.18 Make any changes to the concentration, retention time or peak identification and press F1 to "Save and Quit".
- 5.2.19 If performing a 2 point calibration, Press Enter followed by F1 to highlight the second standard peak area data, F2 to plot the chromatogram, F4 to calculate the peak areas, F9 to save the peak data, F8 to enter the data into the Calibration I.D. File and F1 to Save and Quit.
- 5.2.20 Exit DOS by pressing F10 and copy "Work.PRG" to a filename of choice for the analytical application. Type "Exit" to return to the quantitation screen.
- 5.2.21 Make sure to change the Time Program name in SET1 from "Work" to this new name.
- 5.2.22 Press F3 and change the ID File name from "Dummy" to the appropriate name for the analysis.

**5.3 Re-calibration Procedure**

Follow the procedure above (section 5.2) to perform a re-calibration of an existing calibration file.

**6.0 POST RUN ANALYSIS**

**6.1 Multi-Chromatogram (Post Analysis)**

A stored chromatogram may be re-evaluated after the run is complete by entering the Multi-Chromatogram (Post Analysis) mode. From this screen parameters can be changed and re-plotted. The wavelength of interest can be changed and a spectrum at a selected location in the chromatogram can be generated.

**6.2 3D-Chromatogram (Post Analysis)**



A stored chromatogram may be re-evaluated after the run is complete by entering the 3D-Chromatogram (Post Analysis) mode. From this screen parameters can be changed and re-plotted. The wavelength of interest can be changed and a spectrum at a selected location in the chromatogram can be generated.

### **6.3 Quantitation (Post Analysis)**

The results from a sample chromatogram can be compared to a standard chromatogram and the amount of analyte calculated.

- 6.3.1 Inject the sample into the HPLC and record the resulting chromatogram.
- 6.3.2 After injecting all the samples and saving the chromatograms, enter the quantitation screen by pressing F6 (menu) followed by highlighting the quantitation menu bar and pressing return.
- 6.3.3 Press F1 (SET1) to enter the appropriate sample file name along with other display parameters. Make sure that the Time Program File name is correct.
- 6.3.4 Press F2 (Plot) to plot the specified chromatogram.
- 6.3.5 Press F3 (SET2) to assign the proper integration parameters along with the appropriate ID File name.
- 6.3.6 Press F4 to draw the baseline according to the parameters in SET2 and calculate the resulting peak areas.
- 6.3.7 Press F7 (calculate) to calculate the resulting analyte concentration.

### **7.0 INSTRUMENT SHUT-DOWN**

- 7.1 Make sure that the system controller is in the "Prog" screen and turn off the pumps by pressing "Stop" on the front of the controller.
- 7.2 Turn off the power to the photo diode detector.
- 7.3 Remove the needle from the injection syringe and place the needle into the Rheodyne injector. The seals last longer if the needle is stored in the injector. Replace the red cap over the injector.
- 7.4 Turn off the computer monitor.



**Analytical Operating Procedure**  
**Histochoice in Fixatives**  
**Written By: Warren Belisle**  
**Belisle**

**Document # ACL 10033**  
**Revision Date: 8/5/94**  
**Approved By: Warren**

1. **PURPOSE:**

The purpose of this procedure is to describe the methodology to measure the quantities of Histochoice in fixative solutions. It is assumed that Histochoice is the only aldehyde present in the solution. Otherwise the other aldehydes must be subtracted from the total aldehydes to obtain the Histochoice concentration.

2. **SCOPE:**

Aldehydes react with dinitrophenylhydrazines in an acidic medium to form a dinitrophenylhydrazone complex which absorbs at 357 nanometers. The hydrazones which are formed are quantitated by measuring the absorbance at 357 nm and comparing this to a Histochoice standard of known concentration.

3. **STANDARDS AND SOLUTIONS:**

3.1 **DNPH Reagent**

Dinitrophenylhydrazine	0.025 g
6N HCl q.s. ad	10.0 ml

Outdate: 6 months

3.2 **6N HCl**

HCl Concentrated	50 ml
Deionized H <sub>2</sub> O	50 ml

Caution: Always add the acid to the water. Perform this operation in the fume hood.

Outdate: 6 months

3.3 **Histochoice Working Standard**

Use a sample of Histochoice which is selected to be the working standard and use this without any dilutions.

Concentration = 100% Histochoice  
Store at room temperature in a sealed container.



**Analytical Operating Procedure**  
**Histochoice in Fixatives**

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**4.0 SAMPLE PREPARATION**

Analyze the sample without any dilutions.

**5.0 PROCEDURE**

- 5.1 Prepare duplicate hydrozone derivatives of the Histochoice Working Standard and samples as follows:

Acetonitrile	10.0 ml
Sample or Working Std	0.03ml
DNPH Reagent	0.03 ml

Allow the solutions to stand for 1 hour at room temperature which enables the reaction to go to completion.

- 5.2 Prepare a blank by substituting deionized water for the sample or standard in section 5.1 above.
- 5.3 Turn on the spectrophotometer and adjust the wavelength to 357 nanometers. Allow 30 minutes for the instrument to warm up and equilibrate.
- 5.4 Place the blank solution in a cuvette and zero the instrument.
- 5.3 Measure and record the absorbance of the standard and sample preparations. Record the average of the duplicate readings.

**6.0 Quantitation**

$$\% \text{ Histochoice} = \text{Abs (sample)} / \text{Abs (Standard)} * 100$$

Report the average of the two sample results.

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